α-AMYLASE MUTANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a division of 10/186,042, filed on June 27, 2002, which is a division of 09/672,459, filed on September 28, 2000(now a US Patent No. 6,436,888), which is a continuation of 09/182,859, filed on October 29, 1998 (now US Patent No. 6,143,708), which is a continuation of PCT/DK97/00197 filed April 30, 1997 which claims priority under 35 U.S.C. 119 of Danish applications 0515/96 filed April 30, 1996, 0712/96 filed June 28, 1996, 0775/96 filed July 11, 1996, and 1263/96 filed November 8, 1996, the contents of which are fully incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates, *inter alia*, to novel variants (mutants) of parent Termamyl-like α-amylases, notably variants exhibiting alterations in one or more properties (relative to the parent) which are advantageous with respect to applications of the variants in, in particular, industrial starch processing (e.g. starch liquefaction or saccharification).

BACKGROUND OF THE INVENTION

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides, and there is a very extensive body of patent and scientific literature relating to this industrially very important class of enzymes.

Among more recent disclosures relating to α-amylases, WO 96/23874 provides three-dimensional, X-ray crystal structural data for a Termamyl-like α-amylase which consists of the 300 N-terminal

amino acid residues of the *B. amyloliquefaciens* α-amylase comprising the amino acid sequence shown in SEQ ID No. 4 herein and amino acids 301-483 of the C-terminal end of the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 herein (the latter being available commercially under the tradename Termamyl™), and which is thus closely related to the industrially important *Bacillus* α-amylases (which in the present context are embraced within the meaning of the term "Termamyl-like α-amylases", and which include, *inter alia*, the *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus* α-amylases). WO 96/23874 further describes methodology for designing, on the basis of an analysis of the structure of a parent Termamyl-like α-amylase, variants of the parent Termamyl-like α-amylase which exhibit altered properties relative to the parent.

BRIEF DISCLOSURE OF THE INVENTION

As indicated above, the present invention relates, *inter alia*, to novel α-amylolytic variants(mutants) of a Termamyl-like α-amylase, in particular variants exhibiting altered properties which are advantageous in connection with the industrial processing of starch (starch liquefaction, saccharification and the like).

Alterations in properties which may be achieved in mutants of the invention are alterations in, e.g., substrate specificity, substrate binding, substrate cleavage pattern, thermal stability, pH/activity profile, pH/stability profile [such as increased stability at low (e.g. pH<6, in particular pH<5) or high (e.g. pH>9) pH values], stability towards oxidation, Ca^{2+} dependency, specific activity, and other properties of interest. For instance, the alteration may result in a variant which, as compared to the parent Termamyl-like α-amylase, has a reduced Ca^{2+} dependency

and/or an altered pH/activity profile.

The invention further relates, *inter alia*, to DNA constructs encoding variants of the invention, to methods for preparing variants of the invention, and to the use of variants of the invention, alone or in combination with other α-amylolytic enzymes, in various industrial processes, e.g. starch liquefaction.

DETAILED DISCLOSURE OF THE INVENTION

The Termamyl-like α-amylase

It is well known that a number of α-amylases produced by *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 (commercially available as Termamyl™) has been found to be about 89% homologous with the *B. amyloliquefaciens* α-amylase comprising the amino acid sequence shown in SEQ ID No. 4 and about 79% homologous with the *B. stearothermophilus* α-amylase comprising the amino acid sequence shown in SEQ ID No. 6. Further homologous α-amylases include an α-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the α-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31. Still further homologous α-amylases include the α-amylase produced by the *B. licheniformis* strain described in EP 0252666 (ATCC 27811), and the α-amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like *B. licheniformis* α-amylases are Optitherm™ and Takatherm™ (available from Solvay), Maxamyl™ (available from Gist-brocades/Genencor), Spezym AA™ (available from Genencor), and Keistase™ (available from Daiwa).

Because of the substantial homology found between these α-amylases, they are considered to belong to the same class of α-amylases, namely the class of "Termamyl-like α-amylases".

Accordingly, in the present context, the term "Termamyl-like α-amylase" is intended to indicate an α-amylase which, at the amino acid level, exhibits a substantial homology to Termamyl™, i.e. the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2 herein. In other words, a Termamyl-like α-amylase is an α-amylase which has the amino acid sequence shown in SEQ ID No. 2, No. 4 or No. 6 herein, or the amino acid sequence shown in SEQ ID No. 1 of WO 95/26397 (which amino acid sequence is shown in Fig. 1 and Fig. 2 herein) or in SEQ ID No. 2 of WO 95/26397 (which amino acid sequence is shown in Fig. 2 herein) or in Tsukamoto et al., 1988, (which amino acid sequence is shown in Fig. 2 herein) or i) which displays at least 60%, such as at least 70%, e.g. at least 75%, or at least 80%, e.g. at least 85%, at least 90% or at least 95% homology with at least one of said amino acid sequences and/or ii) displays immunological cross-reactivity with an antibody raised against at least one of said α-amylases, and/or iii) is encoded by a DNA sequence which hybridizes to the DNA sequences encoding the above-specified α-amylases which are apparent from SEQ ID Nos. 1, 3 and 5 of the present application (which encoding sequences encode the amino acid sequences shown in SEQ ID Nos. 2, 4 and 6 herein, respectively), from SEQ ID No. 4 of WO 95/26397 (which DNA sequence, together with the stop codon TAA, is shown in Fig. 1 herein and encodes the amino acid sequence shown in Fig. 1 herein) and from SEQ ID No. 5 of WO 95/26397, respectively.

In connection with property i), the "homology" may be determined by use of any conventional algorithm, preferably by use of the GAP progamme from the GCG package version 7.3 (June 1993)

using default values for GAP penalties [Genetic Computer Group (1991) Programme Manual for the GCG Package, version 7, 575 Science Drive, Madison, Wisconsin, USA 53711].

Property ii) of the α-amylase, i.e. the immunological cross reactivity, may be assayed using an antibody raised against, or reactive with, at least one epitope of the relevant Termamyl-like α-amylase. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g. as described by Hudson et al., 1989. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay, e.g. as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the α-amylases having the amino acid sequences SEQ ID Nos. 2, 4 and 6, respectively, has been found.

The oligonucleotide probe used in the characterization of the Termamyl-like α-amylase in accordance with property iii) above may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the α-amylase in question. Suitable conditions for testing hybridization involve presoaking in 5xSSC and prehybridizing for 1h at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50μg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100μM ATP for 18h at ~40°C, or other methods described by, e.g., Sambrook et al., 1989.

In the present context, "derived from" is intended not only to indicate an α-amylase produced or producible by a strain of the organism in question, but also an α-amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended

to indicate an α-amylase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the α-amylase in question. The term is also intended to indicate that the parent α-amylase may be a variant of a naturally occurring α-amylase, i.e. a variant which is the result of a modification (insertion, substitution, deletion) of one or more amino acid residues of the naturally occurring α-amylase.

Parent hybrid α-amylases

The parent α-amylase may be a hybrid α-amylase, i.e. an α-amylase which comprises a combination of partial amino acid sequences derived from at least two α-amylases.

The parent hybrid α-amylase may be one which on the basis of amino acid homology and/or immunological cross-reactivity and/or DNA hybridization (as defined above) can be determined to belong to the Termamyl-like α-amylase family. In this case, the hybrid α-amylase is typically composed of at least one part of a Termamyl-like α-amylase and part(s) of one or more other α-amylases selected from Termamyl-like α-amylases or non-Termamyl-like α-amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid α-amylase may comprise a combination of partial amino acid sequences deriving from at least two Termamyl-like α-amylases, or from at least one Termamyl-like and at least one non-Termamyl-like bacterial α-amylase, or from at least one Termamyl-like and at least one fungal α-amylase. The Termamyl-like α-amylase from which a partial amino acid sequence derives may, e.g., be any of those specific Termamyl-like αa-

mylase referred to herein.

For instance, the parent α-amylase may comprise a C-terminal part of an α-amylase derived from a strain of *B. licheniformis*, and a N-terminal part of an α-amylase derived from a strain of *B. amyloliquefaciens* or from a strain of *B. stearothermophilus*. For instance, the parent α-amylase may comprise at least 430 amino acid residues of the C-terminal part of the *B. licheniformis* α-amylase, and may, e.g. comprise a) an amino acid segment corresponding to the 37 N-terminal amino acid residues of the *B. amyloliquefaciens* α-amylase having the amino acid sequence shown in SEQ ID No. 4 and an amino acid segment corresponding to the 445 C-terminal amino acid residues of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2, or b) an amino acid segment corresponding to the 68 N-terminal amino acid residues of the *B. stearothermophilus* α-amylase having the amino acid sequence shown in SEQ ID No. 6 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2.

The non-Termamyl-like α-amylase may, e.g., be a fungal α-amylase, a mammalian or a plant α-amylase or a bacterial α-amylase (different from a Termamyl-like α-amylase). Specific examples of such α-amylases include the *Aspergillus oryzae* TAKA α-amylase, the *A. niger* acid α-amylase, the *Bacillus subtilis* α-amylase, the porcine pancreatic α-amylase and a barley α-amylase. All of these α-amylases have elucidated structures which are markedly different from the structure of a typical Termamyl-like α-amylase as referred to herein.

The fungal α-amylases mentioned above, i.e. derived from *A.*

niger and *A. oryzae*, are highly homologous on the amino acid level and generally considered to belong to the same family of α-amylases. The fungal α-amylase derived from *Aspergillus oryzae* is commercially available under the tradename Fungamyl™.

Furthermore, when a particular variant of a Termamyl-like α-amylase (variant of the invention) is referred to - in a conventional manner - by reference to modification (e.g. deletion or substitution) of specific amino acid residues in the amino acid sequence of a specific Termamyl-like α-amylase, it is to be understood that variants of another Termamyl-like α-amylase modified in the equivalent position(s) (as determined from the best possible amino acid sequence alignment between the respective amino acid sequences) are encompassed thereby.

A preferred embodiment of a variant of the invention is one derived from a *B. licheniformis* α-amylase (as parent Termamyl-like α-amylase), e.g. one of those referred to above, such as the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2.

Construction of variants of the invention

The construction of the variant of interest may be accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant. The variant may then subsequently be recovered from the resulting culture broth. This is described in detail further below.

Altered properties of variants of the invention

The following discusses the relationship between mutations which may be present in variants of the invention, and desirable

alterations in properties (relative to those a parent, Termamyl-like α-amylase) which may result therefrom.

Decreased Ca^{2+} dependency

It is highly desirable to be able to decrease the Ca^{2+} dependency of a Termamyl-like α-amylase. Accordingly, one aspect of the invention relates to a variant of a parent Termamyl-like α-amylase, which variant exhibits α-amylase activity and has a decreased Ca^{2+} dependency as compared to the parent α-amylase. Decreased Ca^{2+} dependency will in general have the functional consequence that the variant exhibits a satisfactory amylolytic activity in the presence of a lower concentration of calcium ion in the extraneous medium than is necessary for the parent enzyme. It will further often have the consequence that the variant is less sensitive than the parent to calcium ion-depleting conditions such as those obtained in media containing calcium-complexing agents (such as certain detergent builders).

Decreased Ca^{2+} dependency of a variant of the invention may advantageously be achieved, for example, by increasing the Ca^{2+} binding affinity relative to that of the parent Termamyl-like α-amylase; in other words the stronger the binding of Ca^{2+} in the enzyme, the lower the Ca^{2+} dependency.

It may be mentioned here that WO 96/23874 states that amino acid residues located within 10Å from a sodium or calcium ion are believed to be involved in, or of importance for, the Ca^{2+} binding capability of the enzyme, and that in this connection the mutation N104D [of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2, or an equivalent (N to D) mutation of an equivalent position in another Termamyl-like α-amylase] is contemplated to be of particular interest with respect to decreasing the Ca^{2+} dependency of a Termamyl-like α-amylase.

Other mutations mentioned in WO 96/23874 as being of possible importance in connection with Ca^{2+} dependency include mutations which are contemplated therein to achieve increased calcium binding (and/or thermostability of the enzyme) via stabilization of the C-domain (as defined in WO 96/23874) of the three-dimensional structure of a Termamyl-like α-amylase via formation, for example, of cysteine bridges or salt bridges. Thus, WO 96/23874 discloses that the C-domain of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2 may be stabilized by introduction of a cysteine bridge between domain A and domain C (as defined in WO 96/23874) by introduction of the following mutations:
A349C+I479C and/or L346C+I430C.

WO 96/23874 likewise discloses that a salt bridge may be obtained by introduction of one or more of the following mutations in the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2:

N457D,E
N457D,E + K385R
F350D,E + I430R,K
F350D,E + I411R,K

and that the calcium site of Domain C may be stabilized by replacing the amino acid residues H408 and/or G303 with any other amino acid residue, in particular by introducing one of the substitutions:
H408Q,E,N,D and/or G303N,D,Q,E
which are contemplated to provide better calcium binding or protection from calcium depletion.

(similar mutations in equivalent positions of other Termamyl-like α-amylases being encompassed hereby).

Other substitution mutations (relative to *B. licheniformis* α-amylase, SEQ ID No. 2) which are disclosed in WO 96/23874 as being of apparent importance, *inter alia*, in the context of reducing calcium dependency include the following: R23K, H156Y, A181T, A209V, R214, G310D and P345 (or equivalent mutations in equivalent positions in another Termamyl-like α-amylase).

In the context of the present invention, further substitution mutations which appear to be of importance, inter alia, in relation to reduction of calcium dependency include the following mutations in Domain B (as defined in WO 96/23874):

A181E,D,Q,N,V (which appear to result in shielding of the outermost Ca^{2+} binding site in the junction region between Domain A and Domain B to some extent);

I201(bulkier amino acid), e.g. I201W,F,L (which appear to result in slight alterations in the geometry of the region in the immediate vicinity of the Ca^{2+}-Na^{+}-Ca^{2+} binding site(s) in the junction region between Domain A and Domain B, and in the geometry and/or size of a nearby hole/cavity); and

Y203E,Q (which are believed to result in stronger binding of the outermost Ca^{2+} ion in its binding site in the junction region between Domain A and Domain B);
(or equivalent mutations in equivalent positions in another Termamyl-like α-amylase).

Altered pH optimum (altered pH/activity profile)

WO 96/23874 discloses that it is contemplated to be possible to change the pH optimum of a Termamyl-like α-amylase, or the enzymatic activity thereof at a given pH, by changing the pKa of the active site residues, and that this may be achieved, e.g., by changing the electrostatic interaction or hydrophobic interaction

between functional groups of amino acid side chains of the amino acid residue to be modified and of its close surroundings.

In the context of the present invention, it is believed on the basis of electrostatic considerations [see, e.g., M.K. Gilson, Current Opinion in Structural Biology 5 (1995) pp. 216-223; and B. Honig and A. Nicholls, Science 268 (1995) pp. 1144-1149; and references given therein] and hygroscopicity considerations in relation to the three-dimensional structure of the Termamyl-like α-amylase disclosed in WO 96/23874 that mutations of relevance, *inter alia*, for altering (increasing or decreasing) the pH optimum of a Termamyl-like α-amylase include the following mutations or equivalents thereof [referring here to the sequence of *B. licheniformis* α-amylase (SEQ ID NO 2)]:

Q9K,L,E; F11R,K,E; E12Q; D100N,L; V101H,R,K,D,E,F; V102A,T; I103H,K; N104R,K,D; H105R,K,D,E,W,F; L196R,K,D,E,F,Y; I212R,K,D,E; L230H,K,I; A232G,H,F,S,V; V233D; K234L,E; I236R,K,N,H,D,E; L241R,K,D,E,F; A260S; W263H; Q264R,D,K,E; N265K,R,D; A269R,K,D,E; L270R,K,H,D,E; V283H,D; F284H; D285N,L; V286R,K,H,D,E; Y290R,E; V312R,K,D,E; F323H; D325N; N326K,H,D,L; H327Q,N,E,D,F; Q330L,E; G332D; Q333R,K,H,E,L; S334A,V,T,L,I,D; L335G,A,S,T,N; E336R+R375E; T337D,K; T338D,E; T339D; Q360K,R,E; D365N; G371D,R;

Increased stability at low (acidic) pH

In the context of the present invention, mutations (amino acid substitutions) of importance with respect to achieving increased stability at low pH appear to include mutations corresponding to the following mutations in the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2:

mutations at positions H68, H91, H247, R305, K306, H382, K389, H405, H406, H450 or R483;

the mutations:

H140Y;

H142Y;

H156Y;

H159Y;

H140D+H142R;

H140K+H142D; or

H142Y+H156Y

as well as combinations of any two or more of these mutations.

Increased thermostability and/or altered temperature optimum (altered temperature/activity profile)

A further aspect of the invention relates to a variant of a parent Termamyl-like α-amylase, which variant is the result of one or more amino acid residues having been deleted from, substituted in or added to the parent α-amylase so as to achieve increased thermostability of the variant.

In may be mentioned that in relation to achieving increased thermostability, WO 96/23874 discloses that a particularly interesting variant of a Termamyl-like α-amylase comprises a mutation corresponding to one of the following mutations (using the numbering of the *B. licheniformis* α-amylase amino acid sequence shown in SEQ ID NO 2):

L61W,V,F;

Y62W;

F67W;

K106R,F,W;

G145F,W

I212F,L,W,Y,R,K;

S151 replaced with any other amino acid residue and in particular with F,W,I or L;

R214W;
Y150R,K;
F143W; and/or
R146W.

WO 96/23874 further discloses in this connection that mutations corresponding to one or more of the following mutations in the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2 are of interest in relation to achieving increased thermostability relative to that of the parent α-amylase:

L241I,F,Y,W; and/or
I236L,F,Y,W

L7F,I,W
V259F,I,L
F284W

F350W
F343W

L427F,L,W
V481,F,I,L,W

In the context of the present invention, it can be seen from an alignment of the amino acid sequences of α-amylases from various *Bacillus* species that *B. licheniformis* α-amylase and *B. amyloliquefaciens* α-amylase both contain an "insertion" of three amino acids relative to, e.g., *B. stearothermophilus* α-amylase.

From a model of the structure of *B. licheniformis* α-amylase built on the basis of the three-dimensional structure of the Termamyl-like α-amylase disclosed in WO 96/23784 (*vide supra*),

taking into account the homology of *B. licheniformis* α-amylase to the Termamyl-like α-amylase in question, it can be seen that the above-mentioned "insertion" lies within a part of the structure denoted "loop 8" in WO 96/23784, making this loop bulkier in *B. licheniformis* α-amylase than in the Termamyl-like α-amylase and resulting in a loop that protrudes from the structure, thereby possibly destabilizing the structure. It is therefore contemplated that deletion of one or more amino acids in the region in question in *B. licheniformis* or *B. amyloliquefaciens* α-amylase will improve the thermostability of these α-amylases.

Especially interesting in this connection is deletion of three amino acids within the partial sequence from T369 to I377 (referring to the amino acid sequence of *B. licheniformis* α-amylase shown in SEQ ID No. 2), i.e. the partial sequence: T369-K370-G371-D372-S373-Q374-R375-E376-I377 (or the corresponding partial sequence in *B. amyloliquefaciens* α-amylase). In addition to such deletions, substitution of one or more of the undeleted amino acids within the latter partial sequence may also be advantageous.

Preferable deletions of three amino acids in the partial sequence from T369 to I377 (in the *B. licheniformis* α-amylase) are deletion of K370+G371+D372 (i.e. K370*+G371*+D372*) or deletion of D372+S373+Q374 (i.e. D372*+S373*+Q374*) (or equivalent deletions in the corresponding partial sequence in *B. amyloliquefaciens* α-amylase).

Another type of mutation which would appear to be of value in improving the thermostability of these α-amylases is substitution (replacement) of the entire partial amino acid sequence from T369 to I377 (referring to the sequence of the *B. licheniformis* α-amylase) with one of the following partial sequences of six amino acids (sequence numbering increasing from left to right): I-

P-T-H-S-V; I-P-T-H-G-V; and I-P-Q-Y-N-I (or one of the same substitutions of the corresponding partial sequence in *B. amyloliquefaciens* α-amylase).

Other mutations which can apparently be of some importance in relation to achieving increased thermostability include amino acid substitutions at the following positions (referring to SEQ ID No. 2):

R169 (e.g. R169I,L,F,T);
R173 (especially R173I,L,F,T);
I201F;
I212F;
A209L,T; or
V208I

as well as combinations of any two or more of these mutations.

Increased thermostability at acidic pH and/or at low Ca^{2+} concentration

In the context of the invention, mutations which appear to be of particular relevance in relation to obtaining variants according to the invention having increased thermostability at acidic pH (pH < 7) and/or at low Ca^{2+} concentration include mutations at the following positions (relative to *B. licheniformis* α-amylase, SEQ ID No. 2):

H156, N172, A181, N188, N190, H205, D207, A209, A210, E211, Q264, N265

It may be mentioned here that N and E amino acid residues, respectively, at positions corresponding to N109 and E211, respectively, in SEQ ID No. 2 constitute amino acid residues which are conserved in numerous Termamyl-like α-amylases. Thus, for

example, the corresponding positions of these residues in the amino acid sequences of a number of Termamyl-like α-amylases which have already been mentioned (*vide supra*) are as follows:

Termamyl-like α-amylase	N position	E position
B. licheniformis (SEQ ID No. 2)	N190	E211
B. amyloliquefaciens (SEQ ID No. 4)	N190	E211
B. stearothermophilus (SEQ ID No. 6)	N193	E210
Bacillus NCIB 12512 (WO 95/26397)	N195	E212
Bacillus NCIB 12513 (WO 95/26397)	N195	E212
"*Bacillus* sp. #707" (Tsukamoto et al.)	N195	E212

Mutations of these conserved amino acid residues appear to be very important in relation to improving thermostability at acidic pH and/or at low calcium concentration, and the following mutations are of particular interest in this connection (with reference to the numbering of the *B. licheniformis* amino acid sequence shown in SEQ ID No. 2):

H156Y,D
N172R,H,K
A181T
N188P
N190L,F
H205C
D207Y
A209L,T,V
A210S
E211Q
Q264A,E,L,K,S,T
N265A,S,T,Y

as well as any combination of two or more of these mutations.

An example of a particularly interesting double mutation in this connection is Q264S+N265Y.

Altered cleavage pattern

In the starch liquefaction process it is desirable to use an α-amylase which is capable of degrading the starch molecules into long, branched oligosaccharides, rather than an α-amylase which gives rise to formation of shorter, branched oligosaccharides (like conventional Termamyl-like α-amylases). Short, branched oligosaccharides (panose precursors) are not hydrolyzed satisfactorily by pullulanases, which are used after α-amylase treatment in the liquefaction process, but before addition of a saccharifying amyloglucosidase (glucoamylase). Thus, in the presence of panose precursors, the product mixture present after the glucoamylase treatment contains a significant proportion of short, branched, so-called limit-dextrin, viz. the trisaccharide panose. The presence of panose lowers the saccharification yield significantly and is thus undesirable.

Thus, one aim of the present invention is to arrive at a mutant α-amylase having appropriately modified starch-degradation characteristics but retaining the thermostability of the parent Termamyl-like α-amylase.

It may be mentioned here that according to WO 96/23874, variants comprising at least one of the following mutations are expected to prevent cleavage close to the branching point:

V54L,I,F,Y,W,R,K,H,E,Q
D53L,I,F,Y,W
Y56W
Q333W

G57all possible amino acid residues
A52amino acid residues larger than A, e.g. A52W,Y,L,F,I.

Increased specific activity

In a further aspect of the present invention, important mutations with respect to obtaining variants exhibiting increased specific activity appear to include mutations corresponding to the following mutations in the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2:

mutations (amino acid substitutions) at positions S187 (especially S187D) or Q264 (e.g. Q264R,K,S);

mutations (substitutions) at position Y290 (especially Y290E,K);

the mutation V54I;

as well as combinations of any two or more of the latter mutations, or combinations of one, two or more of the latter mutations with the following multiple mutation: A1*+N2*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I

General mutations in variants of the invention

It may be preferred that a variant of the invention comprises one or more modifications in addition to those outlined above. Thus, it may be advantageous that one or more proline residues present in the part of the α-amylase variant which is modified is/are replaced with a non-proline residue which may be any of the possible, naturally occurring non-proline residues, and which preferably is an alanine, glycine, serine, threonine, valine or leucine.

Analogously, it may be preferred that one or more cysteine

residues present among the amino acid residues with which the parent α-amylase is modified is/are replaced with a non-cysteine residue such as serine, alanine, threonine, glycine, valine or leucine.

Furthermore, a variant of the invention may - either as the only modification or in combination with any of the above outlined modifications - be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID No. 2 is replaced by an Asn and/or Gln, respectively. Also of interest is the replacement, in the Termamyl-like α-amylase, of one or more of the Lys residues present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID No. 2 by an Arg.

It will be understood that the present invention encompasses variants incorporating two or more of the above outlined modifications.

Furthermore, it may be advantageous to introduce point-mutations in any of the variants described herein.

Methods for preparing α-amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of α-amylase-encoding DNA sequences, methods for generating mutations at specific sites within the α-amylase-encoding sequence will be discussed.

Cloning a DNA sequence encoding an α-amylaseCloning a DNA sequence encoding an α-amylaseCloning a DNA sequence encoding an α-amylaseCloning a DNA sequence encoding an α-amylase

The DNA sequence encoding a parent α-amylase may be isolated from any cell or microorganism producing the α-amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α-amylase to be studied. Then, if the amino acid sequence of the α-amylase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify α-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known α-amylase gene could be used as a probe to identify α-amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying α-amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for α-amylase, thereby allowing clones expressing the α-amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to

various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed mutagenesis

Once an α-amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the α-amylase-encoding sequence, is created in a vector carrying the α-amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into α-amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random mutagenesis

Random mutagenesis is suitably performed either as localized or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence in question, or within the whole gene.

WO 96/23874 discloses that in connection with achieving improved binding of a substrate (i.e. improved binding of a carbohydrate species, such as amylose or amylopectin) by a Termamyl-like α-amylase variant, modified (e.g. higher) substrate specificity and/or modified (e.g. higher) specificity with respect to cleavage (hydrolysis) of substrate, the following codon positions for the amino acid sequence shown in SEQ ID NO 2 (or equivalent codon positions for another parent Termamyl-like α-amylase in the context of the invention) appear to be particularly appropriate for targetting:

13-18
50-56
70-76
102-109
163-172
189-199
229-235
360-364
327-335

Improvement of liquefaction performance at low pH and low calcium ion concentration

For an α-amylase to be used in a starch liquefaction process it is of particular interest that it be thermostable and able to function at low pH and low calcium concentrations. In order to improve these properties of a parent Termamyl-like α-amylase, in

particular the *B. licheniformis* α-amylase or a variant or hybrid thereof, random mutagenesis (preferably by use of doped or spiked oligonucleotide primers) followed by appropriate selection of the resulting mutated enzymes may be performed. The direction of selection of regions to randomize and selection of doping are based primarily on stabilization of calcium ions already present, and on improvement in residue/residue or domain/domain electrostatic interactions at low pH. In addition, the regions which have been shown to include positions important for achieving good starch liquefaction performance may be selected.

In order to prepare a variant of a parent Termamyl-like α-amylase having the above properties, at least one of the following regions may advantageously be subjected to random mutagenesis (the numbering of the amino acid residues being as in SEQ ID No. 2):

Region	Residue	Description
I:	153-163	Calcium region between domain A & B, also containing H156
II:	178-192	Calcium region between domain A & B
III:	193-214	Calcium region between domain A & B, also containing A209
IV:	232-237	Calcium region between domain A & B
V:	297-308	Calcium region between domain A & C
VI:	403-409	Calcium region between domain A & C
VII:	428-435	Calcium region between domain A & C
VIII:	131-136	Region containing H133
IX:	164-175	Region in contact with H133 region
X:	262-278	Region containing Q264

Preferably, two, three or four of the above regions are subjected to random mutagenesis in the construction of a novel α-amylase variant of the invention. For instance, the following combinations of regions are suitably subjected to random mutagenesis:

VIII + IX
VIII + IX + II
II + III + IV

IV + I

Furthermore, it is preferred that the mutagenesis is carried out by use of doped or spiked oligonucleotides. The doping is preferably done so as to introduce amino acids contributing to improved stability at low pH and reduced calcium dependency at low pH of the resulting α-amylase variant. Furthermore, when selecting the doping scheme, the possibility of introducing Asn and Gln residues should generally be avoided, since Asn and Gln residues in general are associated with instability at low pH. Preferably, when a Pro residue can be inserted with potential benefits (e.g. as assessed from protein-structural considerations), the doping scheme is prepared to include a preference for introduction of a Pro residue.

The parent Termamyl-like α-amylase to be subjected to random mutagenesis according to the above principle may be any wild type α-amylase or a variant thereof containing one or more mutations. The parent may be a hybrid between at least two α-amylases as explained in further detail herein. Preferably, the parent α-amylase is a mutant of the *B. licheniformis* α-amylase having the sequence shown in SEQ ID No. 2 containing at least one mutation, and preferably multiple mutations. The parent α-amylase may alternatively be a hybrid α-amylase which contains at least a part of the *B. licheniformis* (SEQ ID No. 2) α-amylase. Specific examples of parent α-amylases suited to mutagenesis according to the above-described principles include: variants of the *B. licheniformis* (SEQ ID No. 2) α-amylase which contain at least one of, i.e. one, two, three, four or all five of, the mutations H156Y, A181T, N190F, A209V and Q264S; hybrid α-amylases which contain a part of the *B. licheniformis* (SEQ ID No. 2) α-amylase, preferably a C-terminal part thereof, such as amino acids 35-483 thereof, and a part of another Termamyl-like

α-amylase such as *B. amyloliquefaciens* (SEQ ID No. 4) α-amylase, preferably an N-terminal part thereof such as the first 38 amino acid residues thereof.

In relation to the above, a further aspect of the present invention relates to a method for generating a variant of a parent Termamyl-like α-amylase, which variant exhibits increased stability at low pH and at low calcium concentration relative to the parent, the method comprising:

(a) subjecting a DNA sequence encoding the parent Termamyl-like α-amylase to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing a mutated α-amylase which has increased stability at low pH and low calcium concentration relative to the parent α-amylase.

Step (a) of the latter method of the invention is preferably performed using doped primers, as described in the working examples herein (*vide infra*).

Method of performing random mutagenesis

The random mutagenesis of a DNA sequence encoding a parent α-amylase to be performed in accordance with step a) of the above-described method of the invention may conveniently be performed by use of any method known in the art.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be

performed by use of any combination of these mutagenizing agents.

The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the amylolytic enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase.

Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and mutation in each position is predefined. Furthermore, the doping may be directed to have a preference for the introduction of certain nucleotides, and thereby a preference for introduction of one or more specific amino acid residues. The doping may, e.g., be made so as to allow for the introduction of 90% wild type and 10%

mutations in each position. An additional consideration in choice of doping scheme is genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program (see the working examples herein) which, *inter alia*, ensures that introduction of stop codons is avoided.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent α-amylase enzyme is subjected to PCR under conditions that increase the mis-incorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cereviseae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the amylolytic enzyme by e.g. transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may subsequently be transformed into the expression organism.

The DNA sequence to be mutagenized may conveniently be present in a genomic or cDNA library prepared from an organism expressing the parent amylolytic enzyme. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenizing agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to the expression step (b) or the screening step

(c) being performed. Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gramnegative bacteria such as *E.coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localized random mutagenesis: the random mutagenesis may advantageously be localized to a part of the parent α-amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized random mutagenesis is conveniently performed by use of PCR-generated mutagenesis techniques as described above, or any other suitable technique known in the art.

Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g. by being inserted into a suitable vector, and said part may subsequently be subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

With respect to the screening step in the above-mentioned method of the invention, this may conveniently performed by use of an assay as described in connection with Example 2 herein.

With regard to screening in general, a filter assay based on the following is generally applicable:

A microorganism capable of expressing the mutated amylolytic enzyme of interest is incubated on a suitable medium and under suitable conditions for the enzyme to be secreted, the medium being provided with a double filter comprising a first protein-binding filter and on top of that a second filter exhibiting a low protein binding capability. The microorganism is located on the second filter. Subsequent to the incubation, the first filter comprising enzymes secreted from the microorganisms is separated from the second filter comprising the microorganisms. The first filter is subjected to screening for the desired enzymatic activity and the corresponding microbial colonies present on the second filter are identified.

The filter used for binding the enzymatic activity may be any protein binding filter e.g. nylon or nitrocellulose. The topfilter carrying the colonies of the expression organism may be any filter that has no or low affinity for binding proteins e.g. cellulose acetate or Durapore™. The filter may be pretreated with any of the conditions to be used for screening or may be treated during

the detection of enzymatic activity.

The enzymatic activity may be detected by a dye, fluorescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity.

The detecting compound may be immobilized by any immobilizing agent e.g. agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents.

α-Amylase activity is detected by Cibacron Red labelled amylopectin, which is immobilized on agarose. For screening for variants with increased thermal and high-pH stability, the filter with bound α-amylase variants is incubated in a buffer at pH 10.5 and 60° or 65°C for a specified time, rinsed briefly in deionized water and placed on the amylopectin-agarose matrix for activity detection. Residual activity is seen as lysis of Cibacron Red by amylopectin degradation. The conditions are chosen to be such that activity due to the α-amylase having the amino acid sequence shown in SEQ ID No. 2 can barely be detected. Stabilized variants show, under the same conditions, increased colour intensity due to increased liberation of Cibacron Red.

For screening for variants with an activity optimum at a lower temperature and/or over a broader temperature range, the filter with bound variants is placed directly on the amylopectin-Cibacron Red substrate plate and incubated at the desired temperature (e.g. 4°C, 10°C or 30°C) for a specified time. After this time activity due to the α-amylase having the amino acid sequence shown in SEQ ID No. 2 can barely be detected, whereas variants with optimum activity at a lower temperature will show increase amylopectin lysis. Prior to incubation onto the amylopectin matrix, incubation in all kinds of desired media - e.g. solutions containing Ca^{2+}, detergents, EDTA or other relevant additives - can be carried out in order to screen for changed dependency or for reaction of the

variants in question with such additives.

Testing of variants of the invention

The testing of variants of the invention may suitably be performed by determining the starch-degrading activity of the variant, for instance by growing host cells transformed with a DNA sequence encoding a variant on a starch-containing agarose plate and identifying starch-degrading host cells. Further testing as to altered properties (including specific activity, substrate specificity, cleavage pattern, thermoactivation, pH optimum, pH dependency, temperature optimum, and any other parameter) may be performed in accordance with methods known in the art.

Expression of α-amylase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an α-amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell

genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α-amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* α-amylases mentioned herein comprise a pre-region permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding an α-amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an α-amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely

to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing an α-amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the α-amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The α-amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Industrial Applications

The α-amylase variants of this invention possesses valuable properties allowing for a variety of industrial applications. In particular, enzyme variants of the invention are applicable as a component in washing, dishwashing and hard-surface cleaning detergent compositions. Numerous variants are particularly useful in the production of sweeteners and ethanol from starch, and/or for textile desizing. Conditions for conventional starch- conversion processes, including starch liquefaction and/or saccharification processes, are described in, e.g., US 3,912,590 and in EP patent publications Nos. 252,730 and 63,909.

Production of sweeteners from starch: A "traditional" process for conversion of starch to fructose syrups normally consists of three consecutive enzymatic processes, viz. a liquefaction process followed by a saccharification process and an isomerization process. During the liquefaction process, starch is degraded to

dextrins by an α-amylase (e.g. Termamyl™) at pH values between 5.5 and 6.2 and at temperatures of 95-160°C for a period of approx. 2h. In order to ensure an optimal enzyme stability under these conditions, 1mM of calcium is added (40 ppm free calcium ions).

After the liquefaction process the dextrins are converted into dextrose by addition of a glucoamylase (e.g. AMG™) and a debranching enzyme, such as an isoamylase or a pullulanase (e.g. Promozyme™). Before this step the pH is reduced to a value below 4.5, maintaining the high temperature (above 95°C), and the liquefying α-amylase activity is denatured. The temperature is lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

After the saccharification process the pH is increased to a value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immmobilized glucoseisomerase (such as Sweetzyme™).

At least 3 enzymatic improvements of this process could be envisaged. All three improvements could be seen as individual benefits, but any combination (e.g. 1+2, 1+3, 2+3 or 1+2+3) could be employed:

Improvement 1. Reduction of the calcium dependency of the liquefying alpha-amylase.

Addition of free calcium is required to ensure adequately high stability of the α-amylase, but free calcium strongly inhibits the activity of the glucoseisomerase and needs to be removed, by means of an expensive unit operation, to an extent which reduces the level of free calcium to below 3-5 ppm. Cost savings could be

obtained if such an operation could be avoided and the liquefaction process could be performed without addition of free calcium ions.

To achieve that, a less calcium-dependent Termamyl-like α-amylase which is stable and highly active at low concentrations of free calcium (< 40 ppm) is required. Such a Termamyl-like α-amylase should have a pH optimum at a pH in the range of 4.5-6.5, preferably in the range of 4.5-5.5.

Improvement 2. Reduction of formation of unwanted Maillard products

The extent of formation of unwanted Maillard products during the liquefaction process is dependent on the pH. Low pH favours reduced formation of Maillard products. It would thus be desirable to be able to lower the process pH from around pH 6.0 to a value around pH 4.5; unfortunately, all commonly known, thermostable Termamyl-like α-amylases are not very stable at low pH (i.e. pH < 6.0) and their specific activity is generally low.

Achievement of the above-mentioned goal requires a Termamyl-like α-amylase which is stable at low pH in the range of 4.5-5.5 and at free calcium concentrations in the range of 0-40 ppm, and which maintains a high specific activity.

Improvement 3.

It has been reported previously (US patent 5,234,823) that when saccharifying with *A. niger* glucoamylase and *B. acidopullulyticus* pullulanase, the presence of residual α-amylase activity from the liquefaction process can lead to lower yields of dextrose if the α-amylase is not inactivated before the saccharification stage. This inactivation can typically be carried out by adjusting the pH to below 4.3 at 95°C, before lowering the temperature to

60°C for saccharification.

The reason for this negative effect on dextrose yield is not fully understood, but it is assumed that the liquefying α-amylase (for example Termamyl™ 120 L from *B. licheniformis*) generates "limit dextrins" (which are poor substrates for *B. acidopullulyticus* pullulanase) by hydrolysing 1,4-α-glucosidic linkages close to and on both sides of the branching points in amylopectin. Hydrolysis of these limit dextrins by glucoamylase leads to a build-up of the trisaccharide panose, which is only slowly hydrolysed by glucoamylase.

The development of a thermostable α-amylase which does not suffer from this disadvantage would be a significant process improvement, as no separate inactivation step would be required.

If a Termamyl-like, low-pH-stable α-amylase is developed, an alteration of the specificity could be an advantage needed in combination with increased stability at low pH.

The methodology and principles of the present invention make it possible to design and produce variants according to the invention having required properties as outlined above. In this connection, particularly interesting mutations are mutations in a Termamyl-like α-amylase [for example Termamyl™ itself (*B. licheniformis* α-amylase; SEQ ID No. 2); or a Termamyl-like α-amylase having an N-terminal amino acid sequence (i.e. the partial sequence up to the amino acid position corresponding to position 35 in Termamyl™) which is identical to that in *B. amyloliquefaciens* α-amylase (SEQ ID No. 4), i.e. a Termamyl-like α-amylase having the following N-terminal sequence relative to amino acid sequence of Termamyl™:
A1*+N2*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I, where an asterisk (*) indicates deletion of the amino acid residue in

question] at positions corresponding to any of the following positions in Termamyl™:

H133
H156
A181
A209
G310
H450
V128
N104
V54
S187
H293
A294

(where each of the latter amino acid residues may be replaced by any other amino acid residue, i.e. any other residue chosen among A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y and V), as well as the following triple deletions:

K370*+G371*+D372*
D372*+S373*+Q374*

Particularly preferred substitutions at the above-indicated positions are the following:

H133I
H156Y
A181T
A209V
G310D
H450Y
V128E
N104D
V54W,Y,F,I,L
S187D

H293Y
A294V.

Any combination of one or more (i.e. one, two, three, four..... etc.) of the above indicated mutations may appropriately be effected in a Termamyl-like α-amylase in the context in question, and particularly interesting variants of the invention in the context of achieving one or more of the above-mentioned improvements in relation to the starch liquefaction behaviour of α-amylases include variants comprising combinations of multiple mutations corresponding to the following combinations of mutations in Termamyl™ (SEQ ID No. 2) itself:

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54W+S187D+H293Y+A294V+K370*+G371*+D372*;

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54W+S187D+H293Y+A294V+D372*+S373*+Q374*;

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54Y+S187D+H293Y+A294V+K370*+G371*+D372*;

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54Y+S187D+H293Y+A294V+D372*+S373*+Q374*;

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54F+S187D+H293Y+A294V+K370*+G371*+D372*;

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54F+S187D+H293Y+A294V+D372*+S373*+Q374*;

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54I+S187D+H293Y+A294V+K370*+G371*+D372*;

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54I+S187D+H293Y+A

294V+D372*+S373*+Q374*;

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54L+S187D+H293Y+A294V+K370*+G371*+D372*;

H133I+H156Y+A181T+A209V+G310D+H450Y+V128E+N104D+V54L+S187D+H293Y+A294V+D372*+S373*+Q374*;

Further interesting variants of the invention in this context include variants comprising single or multiple mutations corresponding to the following single or multiple mutations in Termamyl™ itself:

mutations (amino acid substitutions) at positions N172 (e.g. N172R,K), S187 (e.g. S187D), N188 (e.g. N188P), N190 (e.g. N190L,F), H205 (e.g. H205C), D207 (e.g. D207Y), A210 (e.g. A210S), Q264 (e.g. Q264S) or N265 (e.g. N265Y);

the following multiple mutations;
H156Y+A181T+A209V;

H156Y+A181T+N190F+A209V+Q264S

A1*+N2*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+H156Y+A181T+A209V;

A1*+N2*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+H156Y+A181T+N190F+A209V; or

A1*+N2*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+H156Y+A181T+N190F+A209V+Q264S

as well as combinations of any two or more of the latter single or multiple mutations.

As already indicated, numerous variants according to the

invention are particularly well suited for use in starch conversion, e.g. in starch liquefaction. In this connection, a further aspect of the present invention relates to compositions comprising a mixture of:

(i) the α-amylase from *B. licheniformis* having the sequence shown in SEQ ID No. 2 with one or more variants (mutant α-amylases) according to the invention derived from (as the parent Termamyl-like α-amylase) the *B. stearothermophilus* α-amylase having the sequence shown in SEQ ID No. 6; or

(ii) the α-amylase from *B. stearothermophilus* having the sequence shown in SEQ ID No. 6 with one or more variants (mutant α-amylases) according to the invention derived from one or more other parent Termamyl-like α-amylases (e.g. from the *B. licheniformis* α-amylase having the sequence shown in SEQ ID No. 2, or from one of the other parent Termamyl-like α-amylases specifically referred to herein); or

(iii) one or more variants (mutant α-amylases) according to the invention derived from (as the parent Termamyl-like α-amylase) the *B. stearothermophilus* α-amylase having the sequence shown in SEQ ID No. 6 with one or more variants (mutant α-amylases) according to the invention derived from one or more other parent Termamyl-like α-amylases (e.g. from the *B. licheniformis* α-amylase having the sequence shown in SEQ ID No. 2, or from one of the other parent Termamyl-like α-amylases specifically referred to herein).

Preferred mutations in a variant of *B. stearothermophilus* α-amylase to be incorporated in such a mixture include substitutions at N193 and/or at E210, and/or the pairwise deletions R179*+G180* or I181*+G182* (using the numbering of the amino acid sequence for

this particular α-amylase).

Compositions of one of the latter types, containing *B. stearothermophilus* α-amylase or a variant thereof according to the invention, appear to have great potential for use in starch liquefaction. The ratio (expressed, e.g., in terms of mg of active amylolytic protein per liter of liquid medium) between the individual α-amylolytic components of a given mixture will depend on the exact nature and properties of each component.

Detergent Compositions

As mentioned above, variants of the invention may suitably be incorporated in detergent compositions. Reference is made, for example, to WO 96/23874 and WO 97/07202 for further details concerning relevant ingredients of detergent compositions (such as laundry or dishwashing detergents), appropriate methods of formulating the variants in such detergent compositions, and for examples of relevant types of detergent compositions.

Detergent compositions comprising a variant of the invention may additionally comprise one or more other enzymes, such as a lipase, cutinase, protease, cellulase, peroxidase or laccase, and/or another α-amylase.

α-Amylase variants of the invention may be incorporated in detergents at conventionally employed concentrations. It is at present contemplated that a variant of the invention may be incorporated in an amount corresponding to 0.00001-1 mg (calculated as pure, active enzyme protein) of α-amylase per liter of wash/dishwash liquor using conventional dosing levels of detergent.

The present invention is further described with reference to the appended drawing, in which:

Fig. 1 shows the DNA sequence, together with the stop codon TAA, encoding the *Bacillus* strain NCIB 12512 α-amylase described in WO 95/26397, together with the amino acid sequence of the encoded α-amylase (cf. Fig. 2).

Fig. 2 is an alignment of the amino acid sequences of four parent Termamyl-like α-amylases in the context of the invention. The numbers on the extreme left designate the respective amino acid sequences as follows:

1: the amino acid sequence of the *Bacillus* strain NCIB 12512 α-amylase described in WO 95/26397;

2: the amino acid sequence of the *Bacillus* strain NCIB 12513 α-amylase described in WO 95/26397;

3: the amino acid sequence of the *B. stearothermophilus* α-amylase as shown in SEQ ID No. 6 herein;

4: the amino acid sequence of the *Bacillus* sp. #707 α-amylase described by Tsukamoto et al. in Biochem. Biophys. Res. Commun. 151 (1988), pp.25-31.

The numbers on the extreme right of the figure give the running total number of amino acids for each of the sequences in question. Note that for the sequence numbered 3 (corresponding to the sequence in SEQ ID No. 6), the alignment results in "gaps" at the positions corresponding to amino acid No. 1 and No. 175, respectively, in the sequences numbered 1, 2 and 4.
Fig. 3 illustrates the PCR strategy employed in Example 2 (*vide infra*).

MATERIALS AND METHODS

Construction of pSNK101

This *E. coli/Bacillus* shuttle vector can be used to introduce mutations without expression of α-amylase in *E. coli* and then be modified in such way that the α-amylase is active in Bacillus. The vector was constructed as follows: The α-amylase gene in the pX vector (pDN1528 with the following alterations within amyL: BAN(1-33), H156Y, A181T, N190F, A209V, Q264S, the plasmid pDN1528 is further described in Example 1) was inactivated by interruption in the PstI site in the 5´coding region of the alpha-amylase gene by a 1.2 kb fragment containing an *E. coli* origin fragment. This fragment was amplified from the pUC19 (GenBank Accession #:X02514) using the forward primer: 5´-gacctgcagtcaggcaacta-3´ and the reverse primer: 5´-tagagtcgacctgcaggcat-3´. The PCR amplicon and the pX plasmid containing the α-amylase gene were digested with PstI at 37°C for 2 hrs. The pX vector fragment and the *E. coli* origin amplicon were ligated at room temperature. for 1 h and transformed in *E. coli* by electrotransformation. The resulting vector is designated pSnK101.

Fermentation and purification of α-amylase variants

A *B. subtilis* strain harbouring the relevant expression plasmid is streaked on a LB-agar plate with 15 µg/ml

chloramphenicol from -80°C stock, and grown overnight at 37°C. The colonies are transferred to 100ml BPX media supplemented with 15 µg/ml chloramphenicol in a 500 ml shaking flask.

Composition of BPX medium:

Potato starch	100	g/l
Barley flour	50	g/l
BAN 5000 SKB	0.1	g/l
Sodium caseinate	10	g/l
Soy Bean Meal	20	g/l
Na_2HPO_4, 12 H_2O	9	g/l
Pluronic™	0.1	g/l

The culture is shaken at 37°C at 270 rpm for 5 days.

Cells and cell debris are removed from the fermentation broth by centrifugation at 4500 rpm in 20-25min. Afterwards the supernatant is filtered to obtain a completely clear solution. The filtrate is concentrated and washed on a UF-filter (10000 cut off membrane) and the buffer is changed to 20mM Acetate pH 5.5. The UF-filtrate is applied on a S-sepharose F.F. and elution is carried out by step elution with 0.2M NaCl in the same buffer. The eluate is dialysed against 10mM Tris, pH 9.0 and applied on a Q-sepharose F.F. and eluted with a linear gradient from 0-0.3M NaCl over 6 column volumes. The fractions which contain the activity (measured by the Phadebas assay) are pooled, pH was adjusted to pH 7.5 and remaining color was removed by a treatment with 0.5% W/vol. active coal in 5min.

Assay for α-Amylase Activity

α-Amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-coloured starch polymer which has been mixed with bovine serum albumin and a buffer substance and tabletted.

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic

acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM $CaCl_2$, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The α-amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this α-amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolysed by the α-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the α-amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given α-amylase will hydrolyse a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure α-amylase protein) of the α-amylase in question under the given set of conditions.

General method for random mutagenesis by use of the DOPE program

The random mutagenesis may be carried out by the following steps:

1. Select regions of interest for modification in the parent enzyme
2. Decide on mutation sites and nonmutated sites in the selected region
3. Decide on which kind of mutations should be carried out, e.g. with respect to the desired stability and/or performance of the variant to be constructed
4. Select structurally reasonable mutations.
5. Adjust the residues selected by step 3 with regard to step 4.
6. Analyze by use of a suitable dope algoritm the nucleotide

distribution.

7. If necessary, adjust the wanted residues to genetic code realism (e.g. taking into account constraints resulting from the genetic code (e.g. in order to avoid introduction of stop codons))(the skilled person will be aware that some codon combinations cannot be used in practice and will need to be adapted)

8. Make primers

9. Perform random mutagenesis by use of the primers

10. Select resulting α-amylase variants by screening for the desired improved properties.

Suitable dope algorithms for use in step 6 are well known in the art. One algorithm is described by Tomandl, D. et al., Journal of Computer-Aided Molecular Design, 11 (1997), pp. 29-38). Another algorithm, DOPE, is described in the following:

The dope program

The "DOPE" program is a computer algorithm useful to optimize the nucleotide composition of a codon triplet in such a way that it encodes an amino acid distribution which resembles most the wanted amino acid distribution. In order to assess which of the possible distributions is the most similar to the wanted amino acid distribution, a scoring function is needed. In the "Dope" program the following function was found to be suited:

$$s \equiv \prod_{i=1}^{N} \left(\frac{x_i^{y_i}}{y_i^{y_i}} \frac{(1-x_i)^{1-y_i}}{(1-y_i)^{1-y_i}} \right)^{w_i} ,$$

where the x_i's are the obtained amounts of amino acids and groups of amino acids as calculated by the program, y_i's are the wanted amounts of amino acids and groups of amino acids as defined by the user of the program (e.g. specify which of the 20 amino

acids or stop codons are wanted to be introduced, e.g. with a certain percentage (e.g. 90% Ala, 3% Ile, 7% Val), and w_i's are assigned weight factors as defined by the user of the program (e.g. depending on the importance of having a specific amino acid residue inserted into the position in question). N is 21 plus the number of amino acid groups as defined by the user of the program. For purposes of this function 0^0 is defined as being 1.

A Monte-Carlo algorithm (one example being the one described by Valleau, J.P. & Whittington, S.G. (1977) A guide to Mont Carlo for statistical mechanics: 1 Highways. In "Stastistical Mechanics, Part A" Equlibrium Techniqeues ed. B.J. Berne, New York: Plenum) is used for finding the maximum value of this function. In each iteration the following steps are performed:

1. A new random nucleotide composition is chosen for each base, where the absolute difference between the current and the new composition is smaller than or equal to *d* for each of the four nucleotides G,A,T,C in all three positions of the codon (see below for definition of *d*).
2. The scores of the new composition and the current composition are compared by the use of the function s as described above. If the new score is higher or equal to the score of the current composition, the new composition is kept and the current composition is changed to the new one. If the new score is smaller, the probability of keeping the new composition is $\exp\left(1000\left(new\_score - current\_score\right)\right)$.

A cycle normally consists of 1000 iterations as described above in which *d* is decreasing linearly from 1 to 0. One hundred or more cycles are performed in an optimization process. The nucleotide composition resulting in the highest score is finally presented.

EXAMPLE 1

Construction of Termamyl™ variants in accordance with the invention

Termamyl (*B. licheniformis* α-amylase SEQ ID NO. 2) is expressed in *B. subtilis* from a plasmid denoted pDN1528. This plasmid contains the complete gene encoding Termamyl, *amyL*, the expression of which is directed by its own promoter. Further, the plasmid contains the origin of replication, *ori*, from plasmid pUB110 and the *cat* gene from plasmid pC194 conferring resistance towards chloramphenicol. pDN1528 is shown in Fig. 9 of WO 96/23874.

A specific mutagenesis vector containing a major part of the coding region of SEQ ID NO 1 was prepared. The important features of this vector, denoted pJeEN1, include an origin of replication derived from the pUC plasmids, the *cat* gene conferring resistance towards chloramphenicol, and a frameshift-containing version of the *bla* gene, the wild type of which normally confers resistance towards ampicillin (amp^R phenotype). This mutated version results in an amp^S phenotype. The plasmid pJeEN1 is shown in Fig. 10 of WO 96/23874, and the *E. coli* origin of replication, *ori*, *bla*, *cat*, the 5'-truncated version of the Termamyl amylase gene, and selected restriction sites are indicated on the plasmid.

Mutations are introduced in *amyL* by the method described by Deng and Nickoloff (1992, Anal. Biochem. 200, pp. 81-88) except that plasmids with the "selection primer" (primer #6616; see below) incorporated are selected based on the amp^R phenotype of transformed *E. coli* cells harboring a plasmid with a repaired *bla* gene, instead of employing the selection by restriction enzyme digestion outlined by Deng and Nickoloff. Chemicals and enzymes used for the mutagenesis were obtained from the ChameleonÔ mutagenesis kit from Stratagene (catalogue number 200509).

After verification of the DNA sequence in variant plasmids, the truncated gene, containing the desired alteration, is subcloned into pDN1528 as a *Pst*I-*Eco*RI fragment and transformed into the protease- and amylase-depleted *Bacillus subtilis* strain SHA273 (described in WO92/11357 and WO95/10603) in order to express the variant enzyme.

The Termamyl variant V54W was constructed by the use of the following mutagenesis primer (written 5' to 3', left to right):

PG GTC GTA GGC ACC GTA GCC CCA ATC CGC TTG

The Termamyl variant A52W + V54W was constructed by the use of the following mutagenesis primer (written 5' to 3', left to right):

PG GTC GTA GGC ACC GTA GCC CCA ATC CCA TTG GCT CG

Primer #6616 (written 5' to 3', left to right; P denotes a 5' phosphate):

P CTG TGA CTG GTG AGT ACT CAA CCA AGT C

The Termamyl variant V54E was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG TCG TAG GCA CCG TAG CCC TCA TCC GCT TG

The Termamyl variant V54M was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG TCG TAG GCA CCG TAG CCC ATA TCC GCT TG

The Termamyl variant V54I was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG TCG TAG GCA CCG TAG CCA ATA TCC GCT TG

The Termamyl variants Y290E and Y290K were constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGC AGC ATG GAA CTG CTY ATG AAG AGG CAC GTC AAA C

Y represent an equal mixture of C and T. The presence of a codon encoding either Glutamate or Lysine in position 290 was verified by DNA sequencing.

The Termamyl variant N190F was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCA TAG TTG CCG AAT TCA TTG GAA ACT TCC C

The Termamyl variant N188P+N190F was constructed by the use

of the following mutagenesis primer (written 5'-3', left to right):

PCA TAG TTG CCG AAT TCA GGG GAA ACT TCC CAA TC

The Termamyl variant H140K+H142D was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCC GCG CCC CGG GAA ATC AAA TTT TGT CCA GGC TTT AAT TAG

The Termamyl variant H156Y was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCA AAA TGG TAC CAA TAC CAC TTA AAA TCG CTG

The Termamyl variant A181T was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCT TCC CAA TCC CAA GTC TTC CCT TGA AAC

The Termamyl variant A209V was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCTT AAT TTC TGC TAC GAC GTC AGG ATG GTC ATA ATC

The Termamyl variant Q264S was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCG CCC AAG TCA TTC GAC CAG TAC TCA GCT ACC GTA AAC

The Termamyl variant S187D was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGC CGT TTT CAT TGT CGA CTT CCC AAT CCC

The Termamyl variant DELTA(K370-G371-D372) (i.e. deleted of amino acid residues nos. 370, 371 and 372) was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG AAT TTC GCG CTG ACT AGT CCC GTA CAT ATC CCC

The Termamyl variant DELTA(D372-S373-Q374) was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG CAG GAA TTT CGC GAC CTT TCG TCC CGT ACA TAT C

The Termamyl variants A181T and A209V were combined to A181T+A209V by digesting the A181T containing pDN1528-like plasmid (i.e. pDN1528 containing within *amyL* the mutation resulting in the A181T alteration) and the A209V-containing pDN1528-like plasmid (i.e. pDN1528 containing within *amyL* the

mutation resulting in the A209V alteration) with restriction enzyme *Cla*I which cuts the pDN1528-like plasmids twice resulting in a fragment of 1116 bp and the vector-part (i.e. contains the plasmid origin of replication) of 3850 bp. The fragment containing the A209V mutation and the vector part containing the A181T mutation were purified by QIAquick gel extraction kit (purchased from QIAGEN) after separation on an agarose gel. The fragment and the vector were ligated and transformed into the protease and amylase depleted *Bacillus subtilis* strain referred to above. Plasmid from *amy*+ (clearing zones on starch containing agar-plates) and chloramphenicol resistant transformants were analysed for the presence of both mutations on the plasmid.

In a similar way as described above, H156Y and A209V were combined utilizing restriction endonucleases Acc65I and EcoRI, giving H156Y+A209V.

H156Y +A209V and A181T+A209V were combined into H156Y+ A181T+A209V by the use of restriction endonucleases Acc65I and HindIII.

The 35 N-terminal residues of the mature part of Termamyl variant H156Y+ A181T+A209V were substituted by the 33 N-terminal residues of the *B. amyloliquefaciens* α-amylase (SEQ ID NO 4) (which in the present context is termed BAN) by a SOE-PCR approach (Higuchi et al. 1988, Nucleic Acids Research 16:7351) as follows:

Primer 19364 (sequence 5'-3'): CCT CAT TCT GCA GCA GCA GCC GTA AAT GGC ACG CTG

Primer 19362: CCA GAC GGC AGT AAT ACC GAT ATC CGA TAA ATG TTC CG

Primer 19363: CGG ATA TCG GTA TTA CTG CCG TCT GGA TTC

Primer 1C: CTC GTC CCA ATC GGT TCC GTC

A standard PCR, polymerase chain reaction, was carried out using the Pwo thermostable polymerase from Boehringer Mannheim according to the manufacturer's instructions and the temperature cyclus: 5 minutes at 94°C, 25 cycles of (94°C for 30 seconds, 50°C for 45 seconds, 72°C for 1 minute), 72°C for 10 minutes.

An approximately 130 bp fragment was amplified in a first

PCR denoted PCR1 with primers 19364 and 19362 on a DNA fragment containing the gene encoding the *B. amyloliquefaciens* α-amylase.

An approximately 400 bp fragment was amplified in another PCR denoted PCR2 with primers 19363 and 1C on template pDN1528.

PCR1 and PCR2 were purified from an agarose gel and used as templates in PCR3 with primers 19364 and 1C, which resulted in a fragment of approximately 520 bp. This fragment thus contains one part of DNA encoding the N-terminus from BAN fused to a part of DNA encoding Termamyl from the 35th amino acid.

The 520 bp fragment was subcloned into a pDN1528-like plasmid (containing the gene encoding Termamyl variant H156Y+ A181T+A209V) by digestion with restriction endonucleases *Pst*I and *Sac*II, ligation and transformation of the *B. subtilis* strain as previously described. The DNA sequence between restriction sites *Pst*I and *Sac*II was verified by DNA sequencing in extracted plasmids from *amy*+ and chloramphenicol resistant transformants.

The final construct containing the correct N-terminus from BAN and H156Y+ A181T+A209V was denoted BAN(1-35)+ H156Y+ A181T+A209V.

N190F was combined with BAN(1-35)+ H156Y+ A181T+A209V giving BAN(1-35)+ H156Y+ A181T+N190F+A209V by carrying out mutagenesis as described above exept that the sequence of *amyL* in pJeEN1 was substituted by the DNA sequence encoding Termamyl variant BAN(1-35)+ H156Y+ A181T+A209V

Q264S was combined with BAN(1-35)+ H156Y+ A181T+A209V giving BAN(1-35)+ H156Y+ A181T+A209V+Q264S by carrying out mutagenesis as described above exept that the sequence of amyL in pJeEN was substituted by the the DNA sequence encoding Termamyl variant BAN(1-35)+ H156Y+ A181T+A209V

BAN(1-35)+ H156Y+ A181T+A209V+Q264S and BAN(1-35)+ H156Y+ A181T+N190F+A209V were combined into BAN(1-35)+ H156Y+ A181T+N190F+A209V+Q264S utilizing restriction endonucleases *Bsa*HI (*Bsa*HI site was introduced close to the A209V mutation) and *Pst*I.

EXAMPLE 2

Construction, by localized random, doped mutagenesis, of Termamyl-like α-amylas variants having an improved stability at low pH and a reduced dependency on calcium ions for stability compared to the parent enzyme

α-amylases are of great importance for the industrial starch liquefaction process. The variant of the thermostable *B. licheniformis* α-amylase consisting of amino acids 1-33 of the *B. amyloliquefaciens* amylase (SEQ ID NO 4) fused to amino acids 36-483 of the *B. licheniformis* amylase (SEQ ID NO 2) and further comprising the following mutations:
Y156, T181, F190, V209 and S264 (the construction of this variant is described in Example 1)
has a very satisfactory stability at low pH and low calcium concentrations. In an attempt to further improve the stability at low pH and low calcium concentration of said α-amylase variant random mutagenesis in preselected regions wase performed.

The regions were:

Region:	Residue:
I:	Phe153-Thr163
II:	Gln178-Asn192
III:	His205-Arg214
IV:	Ala232-Asp237
and	
VIII:	Gly131-Lys136
IX:	Asp164-Tyr175
X:	Tyr 262-Thr278

Region changed	Total %	Mean %	Number of residues
I:	35	88	8 out of 11
II:	20	86	11 out of 15
III:	27	88	10 out of 10

IV:	34	91	11 out of 12
VIII:	39	86	6 out of 6
IX:	46	93	12 out of 12
X:	27	90	12 out of 13
VIII + IX:	18		
VIII + IX + II:	4		
II + III + IV:	2		
IV + I:	12		

The numbers under Total% give the total number of wild-type (wt) amino acids desired in a given region after doping. The number is obtained by multiplication of the number of mutated positions (e.g. 8 with respect to region I) by their respective wt. With respect to region I the desired total% is 80*80*90*90*90*90*95*90/100 = 35%.

The Mean% is the mean doping level for the total number of positions of the region in question (e.g. 11 positions with respect to region I). For region I the mean% is calculated as follows: 80+80+90+90+90+90+95+90= 705 divided by 11=88%

The DOPE software (see Materials and Methods) was used to determine spiked codons for each suggested change in the seven regions minimizing the amount of stop codons. The exact distribution of nucleotides was calculated in the three positions of the codon to give the suggested population of amino acid changes. The doped regions were doped specifically in the indicated positions to have a high chance of getting the desired residues, but still allow other possibilities.

For instance, the original H156 in the wt sequence was mutated into an Y, meaning a new codon, and then doped 10% for other residues. That is the DNA sequence has the code for a Y instead for a H. In position 156 the Tyr has been programmed to be 90% desired and other residues has been freely allowed. For some positions it was not possible to create the suggested population of amino acid residues because the genetic code restricted the structurally and functionally desired residues. The resulting seven doped oligonucleotides are shown in tables1-

7: with the wt nucleotide and amino acid sequences and the distribution of nucleotides for each doped position. All library primers were synthesized as sense strands.

Table 1: Library DASI (Phe153-Thr163)

153 154 155 156 157 158 159 160 161 162 163
Phe Lys Trp Tyr Trp Tyr His Phe Asp Gly Thr

Primer: 5'CGC GGC AGC ACA TAC AGC GAT T1T 2A3 TGG 45T TGG 67T 8AT TTT GAC GGA A9C GAT TGG GAC GAG TCC CGA AAG3'

Distribution of nucleotides for each doped position.

1: 80% T,20% A.
2: 96% A, 2% G, 2% C.
3: 98% A, 2% T.
4: 93% T, 4% G, 3% A.
5: 97% A, 3% G.
6: 98% T, 2% A.
7: 97% A, 3% C.
8: 90% C, 10% T.
9: 95% C, 5% A.

Table 2: Library DASII (Gln178-Asn192)

178 179 180 181 182 183 184 185 186 187 188 189 190 191 192
Gln Gly Lys Thr Trp Asp Trp Glu Val Ser Asn Glu Phe Gly Asn

Primer: 5'CTG AAC CGC ATC TAT AAG TTT 1A2 34T AAG 567 TGG GAT 89G GA10 GTT A11T 1213T GAA T1415 161718 AAC TAT GAT TAT TTG ATG TAT3'

Distribution of nucleotides for each doped position.

1: 93% C, 7% A.

2: 84% G, 16% T.

3: 95% G, 5% A.

4: 95% G, 5% C.

5: 94% A, 6% G.

6: 95% C, 5% A.

7: 62% T, 38% G.

8: 87% T, 13% A.

9: 91% G, 9% C.

10: 92% G, 8% T.

11: 90% G, 5% A, 5% C.

12: 88% A, 12% C.

13: 88% A, 12% C.

14: 93% T, 5% A, 2% C.

15: 97% T, 3% G.

16: 86% G, 14% A.

17: 89% G, 11% C.

18: 60% G, 40% T.

Table 3: Library DASIII (His205-Arg214)

205 206 207 208 209 210 211 212 213 214

His Pro Asp Val Val Ala Glu Ile Lys Arg

Primer: 5'TAT GCC GAC ATC GAT TAT GAC 12T 3CT 456 7TT 8910 1112T 13A14 15T16 A17A 1819A TGG GGC ACT TGG TAT GCC AAT 3'

Distribution of nucleotides for each doped position.

1: 89% C, 11% T.

2: 89% A, 11% G.

3: 95% C, 2.5 %T, 2.5% A.

4: 96% G, 1% A, 3% T.

5: 96% A, 4% C.

6: 98% T, 2% A.

7: 95% G, 2.5% A, 2.5% C.

8: 93% G, 7% A.

9: 96% T, 4% A.

10: 84% A, 16% G.

11: 81% G, 7% A, 7% T, 5% C.

12: 98% C, 2% A.

13: 96% G, 4% C.

14: 94% G, 6% T.

15: 82% A, 18% T.

16: 50% A, 50% T.

17: 90% A, 10% G.

18: 70% A, 30% C.

19: 86% G, 14% A

Table 4: Library DASIV (Ala232-Asp243)

232	233	234	235	236	237	238	239	240	241	242	243
Ala	Val	Lys	His	Ile	Lys	Phe	Ser	Phe	Leu	Arg	Asp

Primer: 5'TTG GAC GGT TTC CGT CTT GAT 12T G3T AAA 456 7TT A8G T9T 1011T T12T 13T14 1516G GA17 TGG GTT AAT CAT GTC AGG GAA

Distribution of nucleotides for each doped position.

1: 93% G, 3.5% A, 3.5% T.

2: 94% C, 4% T.

3: 94% T, 6% C.

4: 93% C, 2% T, 2% A, 3% G.

5: 98% A, 2% T.

6: 98% T, 2% A.

7: 95% A, 5% C.

8: 94% A, 6% G.

9: 90% T, 10% A.

10: 89% T, 11% A.

11: 89% C, 11% A.

12: 95% T, 5% A

13: 64% C, 33% T, 3% A.

14: 93% A, 7% T.

15: 90% A, 10% C.
16: 90% G, 5% A, 5% C
17: 90% T, 10% A

Table 5: Library DASVIII (Gly131-Lys136)

131 132 133 134 135 136
Gly Glu His Leu Ile lys

Primer: 5' GCT GAC CGC AAC CGC GTA ATT TCA 123 GA4 56T 78A 9TA A10G GCC TGG ACA CAT TTT CAT TTT 3'

Distribution of nucleotides for each doped position.

1: 91% G, 9% A.
2: 87% G, 13% C.
3: 90% T, 10% G.
4: 90% G, 10% T.
5: 85% C, 8% T, 7% A.
6: 89% A, 9% T, 2% C.
7: 88% T, 12% A
8: 88% T, 11% C, 1% G
9: 92% A, 8% T
10: 93% A, 7% G

Table 6: Library DASIX (Asp164-Tyr175)

164 165 166 167 168 169 170 171 172 173 174 175
Asp Trp Asp Glu Ser Arg lys Leu Asn Arg Ile Tyr

Primer: 5'TGG TAC CAT TTT GAC GGA ACC GAT TGG 1A2 GAG 3CG CGA A4G 56A A7T A8G 9 1011 T12T AAG TTT CAA GGA AAG GCT TGG 3'

Distribution of nucleotides for each doped position.

1: 94% G, 6% A.

2: 96% T, 4% G.
3: 92% T, 4% A, 4% G.
4: 95% A, 5% G.
5: 93% C, 7% A.
6: 92% T, 8% A.
7: 90% A, 5% G,5% C.
8: 90% G, 10% A.
9: 92% A, 6% G, 2% T.
10: 92% T, 8% A.
11: 50% T, 50% C.
12: 96% A, 4% T.

Table 7: Library DASX (Tyr262-Asn278)

262 263 264 265 266 267 268 269 270 271 272 273 374 275 276 277 278
Tyr Trp Ser Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn

Primer: 5'GAA ATG TTT ACG GTA GCT GAA T1T TGG 234 56T 7A8 91011 1213T 1415T 16T17 GA18 A19T T20T 21T22 A23C A24G ACA 25AT TTT AAT CAT TCA GTG TTT GAC3'

Distribution of nucleotides for each doped position.
1: 95% A, 5% T.
2: 97% A, 3% G.
3: 95% G, 2.5% A, 2.5% C.
4: 94% T, 6.2% G.
5: 97% A, 3% T.
6: 94% A, 3% G, 3% C.
7: 95% G, 5% A.
8: 95% T, 5% A.
9: 52% T, 45% C, 3% A.
10: 96% T,4% C.
11: 60% A, 40% G.
12: 90% G, 10% A.

13: 94% G, 6% C.

14: 81% G, 8% A, 8% T, 3% C.

15: 98% C, 2% T.

16: 90% C, 10% A.

17: 50% G, 50% T.

18: 90% A, 10% T.

19: 90% A, 5% G, 5% C.

20: 95% A, 5% T.

21: 91% T,9% A.

22: 92% A, 8% G.

23: 94% A, 3% G, 3% C.

24: 93% G, 7% A.

25: 90% A, 10% G.

Random mutagenesis

The spiked oligonucleotides apparent from Tables 1-7 (which by a common term is designated FDAS in Fig. 3) and reverse primers RDAS for each region and specific B. licheniformis primers covering the SacII and the SalI sites are used to generate PCR-library-fragments by the overlap extension method (Horton et al., Gene, 77 (1989), pp. 61-68) with an overlap of 21 bp. Figure 3 shows the PCR strategy. The PCR fragments are cloned in the *E. coli/Bacillus* shuttle vector pSNK101 (see Materials and Methods) enabling mutagenesis in *E. coli* and immediate expression in *Bacillus subtilis* preventing lethal accumulation of amylases in *E. coli*. After establishing the cloned PCR fragments in E. coli, a modified pUC19 fragment is digested out of the plasmid and the promoter and the mutated Termamyl gene is physically connected and expression can take place in *Bacillus*.

Screening

The seven libraries may be screened in the low pH and the low calcium filter assays described below.

Low pH filter assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with 10 µg/ml chloramphenicol at 37°C for at least 21 hrs. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with citrate buffer, pH 4.5 and incubated at 80°C for 15 min. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on plates containing 1% agarose, 0.2% starch in citrate buffer, pH 6.0. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hrs. at 50°C. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

Low Calcium filter assay

The assay is performed in the same way as the low pH filter assay with the following modifications: The filter with bound proteins is incubated at 95°C, pH 6.0 for 1 h. with different EDTA concentrations (0.001 mM - 100 mM).

The following variants were obtained by the above method (BAN designates *B. amyloliquefaciens* α-amylase):

* BAN/Termamyl hybrid *+ H156Y+A181T+N190F+ A209V+Q264S + **E211Q**
* BAN/Termamyl hybrid *+ H156Y+A181T+N190F+ A209V+Q264S + **H205C + D207Y + A210S**

The mutations indicated in bold were introduced by the

random mutagenesis method. The stability data for these variants appear from Table 11 in Example 3.

In an analogous manner to that described above, random mutagenesis of the above identified seven regions are performed on the parent *B. licheniformis* α-amylase (SEQ ID NO 2). The doping scheme is determined analogously to that used above.

EXAMPLE 3

Measurement of the calcium- and pH-dependent stability

Normally, the industrial liquefaction process runs using pH 6.0-6.2 as liquefaction pH and an addition of 40ppm free calcium in order to improve the stability at 95°C-105°C . Some of the herein proposed substitutions have been made in order to improve the stability at

1. lower pH than pH 6.2 and/or
2. at free calcium levels lower than 40ppm free calcium.

Three different methods have been used to measure the improvements in stability obtained by the different substitutions in Termamyl:

1. One assay which measures the stability at slightly reduced pH, pH 5.5, in the presence of 40ppm free calcium. (thereby, the improvement of stability at low pH is measured). 10 µg of the variant were incubated under the following conditions: A 0.1 M acetate solution, pH adjusted to pH 5.5, containing 40ppm calcium and 5% w/w common corn starch (free of calcium). Incubation was made in a water bath at 95°C for 30 minutes.
2. Another assay which measure the stability in the absence of free calcium and where the pH is maintained at pH 6.2. This assay measures the decrease in calcium sensitivity:

10 µg of the variant were incubated under the following conditions: A 0.1 M acetate solution, pH adjusted to pH 6.2, containing 5% w/w common corn starch (free of calcium). Incubation was made in a water bath at 95°C for 30 minutes.

1. A third assay wherein the conditions of assays nos. 1 and 2 have been combined. This assay measures the stability in the absence of calcium and at low pH (pH 5.5).
2. A fourth assay similar to no.3. where the pH has been further reduced to pH 5,0

Stability determination

All the stability trials 1,2, 3 and 4 have been made using the same set up. The method was:

The enzyme was incubated under the relevant conditions (1-4). Samples were taken at 0, 5, 10, 15 and 30 minutes and diluted 25 times (same dilution for all taken samples) in assay buffer (0.1M 50mM Britton buffer pH 7.3) and the activity was measured using the Phadebas assay (Pharmacia) under standard conditions pH 7.3, 37°C.

The activity measured before incubation (0 minutes) was used as reference (100%). The decline in percent was calculated as a function of the incubation time. The table shows the residual activity after 30 minutes of incubation.

Specific activity determination.

The specific activity was determined using the Phadebas assay (Pharmacia) as activity/mg enzyme. The activity was determined using the α-amylase assay described in the Materials and Methods section herein.

Results:

Table 8: Stability method no.1. / Low pH stability improvement

Variant	**residual activity after 30min. of incubation**	**Specific activity, 37°C, pH 7.3. Percent relative to Termamyl**
Termamyl wt.	5%	100%
H156Y	15%	100%
A209V	18%	100%

Q264S	30%	130%
H156Y+A181T+ A209V	39%	100%
H133Y+H156Y+ A181T+A209V+ H293Y+A294V+ G310D+H450Y	58%	85%
BAN/Termamyl hybrid* + H156Y+A181T+ A209V	48%	115%

Table 9: Stability method no.2. / decreased calcium sensitivity

Variant	residual activity after 30min. of incubation	Specific activity, 37°C, pH 7.3. Percent relative to Termamyl
Termamyl wt.	52%	---
H156Y	70%	---
A209V	75%	---
A181T	57%	115%
N190F	34%	140%
N190F + N188P	57%	190%

Table 10: Stability method no.3. /Low pH stability improvement + decreased calcium sensitivity

Variant	residual activity after 30min. of incubation	Specific activity, 37°C, pH 7.3 Units/mg enzyme
Termamyl wt.	3%	---
BAN/Termamyl hybrid* + H156Y+A181T+ A209V	20%	---
Q264S	5%	---
H140K+H142D	5%	115%
V128E	50% **	115%
BAN/Termamyl hybrid *+ H156Y+A181T+ N190F+ A209V	39%	170%
BAN/Termamyl hybrid *+ H156Y+A181T+ A209V+Q264S	29%	175%
BAN/Termamyl hybrid *+ H156Y+A181T+N190F+A209V+Q264S	57%	210%

* BAN (*B. amyloliquefaciens* α-amylase (SEQ ID NO 4)/Termamyl (*B. licheniformis* α-amylase (SEQ ID NO 2) PCR hybrid.
First 33 N-terminal aminoacids are BAN and the rest 36-483 are Termamyl (the construction of the variant is described in Example 1).

** Measured after 5min. of incubation. Compared to Termamyl wt.

Which under same conditions shows 36% residual activity.

Table 11: Stability method no.4. /Low pH stability improvement (pH 5,0) + decreased calcium sensitivity

Variant	residual activity after 30min. of incubation	Specific activity, 37°C, pH 7.3 Units/mg enzyme
BAN/Termamyl hybrid *+ H156Y+A181T+N190F+ A209V+Q264S	9%	210%
BAN/Termamyl hybrid *+ H156Y+A181T+N190F+ A209V+Q264S + **E211Q**	28%	160%
BAN/Termamyl hybrid *+ H156Y+A181T+N190F+ A209V+Q264S + **H205C + D207Y + A210S**	33%	130%

* as indicated in relation to Table 10

The variants in the above Table 11 were constructed by means of the localized random mutagenesis described in Example 2.

EXAMPLE 5

α-amylase stability at low pH and high temperature

This example summarises the stability results of variants characterised by a fluorimetric assay at 70°C under two different conditions, (1) pH 4.5 and 1 mM $CaCl_2$ and (2) pH 6.2 and 10 μM $CaCl_2$.

Description of method

All fluorescence experiments were performed on a Perkin-Elmer LS-50 luminescence spectrometer using a 4-cuvette holder. The temperature was controlled by a circulating water-bath and measured directly in the cuvette using a Noronix Digital Thermometer (model NTD 100). During measurements, thorough mixing of reagents in the

cuvette was ensured using magnetic stirrers operating at high stirring rate. The cuvettes were capped with teflon-lids to minimize evaporation.

Intrinsic protein-fluorescence (due to Trp side-chains) was monitored by excitation at 280 nm and emission at 350 nm. Slit-widths were 5 nm.

During kinetic measurements, 4 reactions were monitored in parallel. Data was collected in the *Wavelength Programme* dialogue, allowing automatic data-collection over a prolonged period (*e.g.* over an hour).

Unfolding was carried out at 70°C. Unfolding conditions were
(1) 50 mM NaOAc pH 4.5 and 1 mM $CaCl_2$
(2) 50 mM NaOAc pH 6.2 and 10 μM $CaCl_2$.
Protein concentration was 5 μg/ml and glycerol was at 0.5% w/v (from protein stock solution).

Note: There was some variation from day to day in the absolute value of the unfolding half times due to slight temperature variations (occasioned by *e.g.* different amounts of water in the water bath). However, Termamyl was always included as one of the four enzymes analyzed in each experiment, in effect making it an internal standard. Unfolding rates relative to this internal standard were satisfactorily reproducible (performed in triplicate).
Data analysis was carried out using *GraphPad Prism* software.
At pH 4.5, unfolding data could be fitted very satisfactorily to a single-exponential decay with drift:

$$F(t) = A*\exp(-ln(2)*t/t_{½}) + drift*t + offset \qquad (1)$$

where F is the measured fluorescence, A is the amplitude of the unfolding, t is time and $t_{½}$ is the half-time of unfolding.

At pH 6.2, unfolding was more complex (involving an initial lag phase), and data could not be fitted to eq. 1. Instead, the time taken for the fluorescence signal to decay to 50% of the initial signal was used as an apparent $t_{½}$.

From these half-times, the change in free energy of unfolding

relative to that of Termamyl could be calculated as follows:

$$DDG = R*T*ln(t_{½}^{mutant} / t_{½}^{Termamyl}) \qquad (2)$$

where R is the universal gas constant and T is the temperature (the value of $R*T$ is 0.5919, giving a DDG value in kcal/mol).

By converting data to DDG values, the destabilizing/stabilizing effects of different mutations can be compared directly and examined for additivity ($DDG_{1+2} = DDG_1 + DDG_2$) and synergy ($DDG_{1+2} > DDG_1 + DDG_2$) where DDG_{1+2} is the energy-effect of introducing mutations 1 and 2.

Results

Unfolding of amylases at low pH and high temperature may be followed by the decay in Trp-fluorescence. At pH 4.5 and 1 mM $CaCl_2$, all amylases unfold fairly rapidly.

The unfolding data at pH 4.5 fit better to a double-exponential equation than to a single-exponential equation. However, since the second phase is very slow, it is approximated by a linear drift (equation 1). Unfolding at pH 6.2 and 10 μM $CaCl_2$ at 70°C is much less rapid than at pH 4.5 despite the low $[Ca^{2+}]$. Unfolding is far from complete within an hour and it is not possible to fit the data to a single-exponential equation. Instead, the time taken for the fluorescence signal to decay to 50% of the initial signal is used as an apparent $t_{½}$.

Results of the fluorescence assay are presented in Table 12.

Table 12

Summary of data for unfolding of Termamyl variants pH 4.5 and pH 6.2 at 70°C.

At pH 4.5, $t_{½}^{Termamyl}$ = 200 s; at pH 6.2, $t_{½}^{Termamyl}$ = 2800 s.

$DDG = -RT*ln(t_{½}^{Termamyl}/t_{½}^{mutant})$

	pH 4.5, 1 mM $CaCl_2$		pH 6.2, 10 μM $CaCl_2$	
Mutation	*DDG* (kcal/mol)	$t_{½}/t_{½}^{Termamyl}$	*DDG* (kcal/mol)	$t_{½}/t_{½}^{Termamyl}$
Wildtype	0	1.0	0	1.0
A209V	-0.36	1.85	-0.72	3.39
H133Y+A209V	-0.77	3.67	-0.61	2.78
H156Y	0.06	0.90	-0.10	1.18
A181T	-0.06	1.10	-0.28	1.60
A181T+A209V	-0.44	2.09	< -1 (73%) [a]	> 5
S187D	0.37	0.54	0.19	0.72
H450Y	-0.49	2.29	0.15	0.78
L270D	-0.35	1.8	-0.10	1.2
A181T+H156Y	-0.17	1.34	-0.62	2.84
H133I	-0.33	1.75	-0.42	2.02
H133Y+H156Y+A181T+A209V+H293Y+A294V+G310D+H450Y	-0.96	5.10	< -1 (58%) [a]	>5
V128E	-0.10	1.2	-0.25	1.5
H156Y+A181T+A209V	-0.32	1.71	-0.49	2.30
H156Y+A181T+A209V+H450Y	-0.42	2.05	-0.63	2.92
H156Y+A181T+A209V+H450Y+H133I	-0.81	3.9	< -1 (65%) [a]	> 5
H156Y+A181T+A209V+H133I	-0.70	3.3	< -1 (77%) [a]	> 5
Q264S	-0.26	1.6	-0.14	1.3
H156Y+A181T+A209V+	-0.43	2.1	-0.82	4.0

Delta(1,2)+ L3V+M15T+ R23K+S31A+A32E+Y33H+A35S+E36D+H37I				
Q264S+N265Y	-0.33	1.8	- 0.07	1.1
Q264S+N265Y+N190F	-1.07	6.1	-0.67	3.1
Q264S+N265Y+N190F+ H133I+A209V	-1.66	16.5	<-1(82%)[a]	> 5
H156Y+A181T+A209V+ Delta(1,2)+ L3V+M15T+ R23K+S31A+A32E+Y33H+ A35S+E36D+H37I+N190F	-0.30	1.7	<-1(66%) [a]	> 5
H156Y+A181T+A209V+Delta(1,2) +L3V+M15T+ R23K+S31A+A32E+Y33H+ A35S+E36D+H37I+Q264S	-0.43	2.1	-0.86	4.3
H156Y+A181T+A209V+Delta(1,2)+ L3V+M15T+ R23K+S31A+A32E+Y33H+ A35S+E36D+H37I Q264S+N190F	-0.36	1.8	<-1(76%) [a]	> 5
H156Y+A181T+A209V+N190F+Q264S	-1.3	8.6	< -1 (66%) [a]	> 5

[a] The percentage indicates the level to which the initial fluorescence level had declined in the course of 3 hours at 70°C. The slow decline is indicative of high stability.

EXAMPLE 5

α-amylase variants with increased specific activity

This example summarises the results of variants characterised by having increased specific activity compared to

Termamyl wt. The presence of these substitutions either in combination with each other or as single substitutions added to stabilising substitutions increases the specific activity of the resulting variant. The specific activity was determined using the α-amylase (Phadebas) assay described in the Materials and Methods where the activity/mg enzyme was determined. The activity was determined using the following description where the pH was 7.3, temperature 37°C and testing time 15min. and buffer as defined.

MUTATION	SPEC.ACTIVITY (PHADEBAS ASSAY), INDEX RELATIVE TO TERMAMYL WT.
S187D	260%
V54I	160%
BAN/Termamyl hybrid: (Δ(1,2)+L3V+M15T+R23K+S31A+A32E+Y33H+A35S+E36D+H37I)	140%
Δ(D372+S373+Q374)	125%
Δ(K370+G371+D372)	125%
BAN/Termamyl hybrid: (Δ(1,2)+L3V+M15T+R23K+S31A+A32E+Y33H+A35S+E36D+H37I)+Δ(D372+S373+Q374)+ V54I+S187D	360%
Q264S	130%
Y290E	155%
Y290K	140%
N190F	150%

EXAMPLE 6

Testing of specificity variants (saccharification)

It has been reported previously (US patent 5,234,823) that, when saccharifying with glucoamylase and pullulanase, the

presence of residual α-amylase activity arising from the liquefaction process, can lead to lower yields of glucose, if the α-amylase is not inactivated before the saccharification stage. This inactivation can be typically carried out by adjusting the pH to below 4.3 at 95°C, before lowering the temperature to 60°C for saccharification.

The reason for this negative effect on glucose yield is not fully understood, but it is assumed that the liquefying a-amylase (for example Termamyl 120 L from *B.licheniformis*) generates "limit dextrins" (which are poor substrates for pullulanase), by hydrolysing 1,4-alpha-glucosidic linkages close to and on both sides of the branching points in amylopectin. Hydrolysis of these limit dextrins by glucoamylase leads to a build up of the trisaccharide panose, which is only slowly hydrolysed by glucoamylase.

The development of a thermostable α-amylase, which does not suffer from this disadvantage would be a significant improvement, as no separate inactivation step would be required.

A number of *B.licheniformis* α-amylase variants, with altered specificity, were evaluated by saccharifying a DE 10 Maltodextrin substrate with *A.niger* glucoamylase and *B.acidopullulyticus* pullulanase under conditions where the variant amylase was active.

The saccharification reactions were monitored by taking samples at 24 hour intervals and analysing them by HPLC. The standard reaction conditions were:

Substrate concentration	28.2% w/w
Temperature	60°C
Initial pH (at 60°C)	4.7
Enzyme dosage	
Glucoamylase	0.18 AG/g DS
Pullulanase	0.06 PUN/g DS
α-amylase	60 NU/g DS

The following enzymes were used:

Glucoamylase: AMG (Novo Nordisk) 153 AG/g

Pullulanase: Promozyme (Novo Nordisk) 295 PUN/g

α-amylase:

Termamyl (Novo Nordisk)	135 KNU/g
V54Y	313 KNU/g
A52W	5,116 NU/ml
D53E	3,280 NU/ml
D53W	599 NU/ml
A52W+V54Y	134 NU/ml

The mutations listed in the α-amylase list above are used to indicate variants of the *B. licheniformis* α-amylase (SEQ ID NO 2) (Termamyl) which has been modified by the indicated mutation(s).

Substrates for saccharification were prepared by dissolving 230g DE 10 spray-dried maltodextrin, prepared from common corn starch, in 460 ml boiling deionized water and adjusting the dry substance to approximately 30% w/w. The pH was adjusted to 4.7 (measured at 60°C) and aliquots of substrate corresponding to 15g dry weight, were transferred to 50ml blue cap glass flasks.

The flasks were then placed in a shaking water bath equilibrated at 60°C, and the enzymes added. The pH was readjusted to 4.7 where necessary. 2 ml samples were taken periodically, the pH adjusted to about 3.0, and then heated in a boiling water bath for 15 minutes to inactivate the enzymes. After cooling, the samples were treated with approximately 0.1g mixed bed ion exchange resin (BIO-Rad 501 X8 (D)) for 30 minutes on a rotary mixer to remove salts and soluble N. After filtration, the carbohydrate composition was determined by HPLC. After 72 hours, the following results were obtained:

Added α-amylase	% DP_1	% DP_2	% DP_3	% DP_{4+}

None (control)	96.59	2.2	0.3	1.0
V54Y	96.5	2.2	0.4	0.9
A52W+V54Y	96.4	2.2	0.5	0.9
Termamyl	96.3	2.1	0.8	0.8

Compared with the control (no active α-amylase present during liquefaction), the presence of active α-amylase variants V54Y and A52W+V54Y did not lead to elevated panose levels (DP_3)..

If these α-amylase variants are used for starch liquefaction, it will not be necessary to inactivate the enzyme before the commencement of saccharification.

EXAMPLE 7

Evaluation of B.licheniformis variants under simulated liquefaction conditions

The standard process for industrial starch liquefaction comprises two stages, normally referred to as primary and secondary liquefaction. In the first stage, a 30-40% w/w starch slurry at pH 5.5-6.0, to which has been added a thermostable alpha-amylase from B. licheniformis or B. stearothermophillus, is heated to 105-110°C in a jet cooker where live steam is injected into the starch stream. After a holding time of 5-10 minutes under pressure at this temperature, the liquefied starch is flash cooled to about 95°C and held at that temperature for 60-120 minutes.

In order to evaluate small quantities of enzyme on a laboratory scale the following test method was used:

10g aliquots of a suspension of common corn starch (Cerestar GL 3406) in deionized water (approx. 30% w/w) are weighed out into 100 ml conical flasks (Schott GL 125) which are fitted with tight fitting screw caps. The pH, calcium level and enzyme dosage in the suspension can be varied.

4 flasks are used for each different set of experimental conditions. The flasks are placed in a shaking oil-bath (Heto VS 01) maintained at 105°C. After a period of 7 minutes, cold oil is poured into the bath to lower the temperature to 95 °C. For each experimental series, flasks are removed after 20, 40, 60 and 90 minutes and immediately cooled under running water. One drop of 1N HCL is added to each flask to inactivate the enzyme.The reaction is monitored by measuring the DE (reducing sugar content expressed as glucose) using the Neocuproine method.

The details of this method can be found in "Determination of reducing sugar with improved precision. Dygert, Li, Florida and Thomas, Anal Biochem, 13, 368 (1965).

The following DEs were recorded after 90 minutes

pH 6.0
5 ppm calcium added
10.9mg enzyme protein /g starch

I.D. no.	Termamyl	Hybrid+ H156Y+ A181T+ A209V+ N190F+Q264S	Hybrid+ H156Y+ A181T+ A209V+ N190F	H156Y+ A181T+ A209V+N190F+ Q264S	Hybrid+ H156Y+ A181T+ A209V+ Q264S	Hybrid+ H156Y+ A181T+ A209V	Q264S	Q264S+N265Y	Q264S+N265Y+ N190F	H156Y+ A181T+ A209V
045-96		16,0	16.9	13.2						
038-96	6.5	13.9								
035-96			15.2		12.9	9.9				
033-96							6.7	7.2	12.1	
031-96	4.5						7.0	8.8	12.5	
029-96	4.0					8.7	5.2			7.7
039-96		14.9	16.3		14.4					

- **Hybrid = BAN/Termamyl PCR hybrid as described in Example 3**

pH 6.0
40 ppm calcium added
10.9mg enzyme protein
/g starch

I.D. no.	Termamyl
045-96	12.6
007-97	12.1

pH 5.5
5 ppm calcium added
10.9mg enzyme protein
/g starch

I.D. no.	Hybrid+ H156Y+ A181T+ A209V+ N190F+Q264S	Hybrid+ H156Y+ A181T+ A209V+ N190F	H156Y+ A181T+ A209V+N190F+Q264S
001-97	14.8	15.2	12.6

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